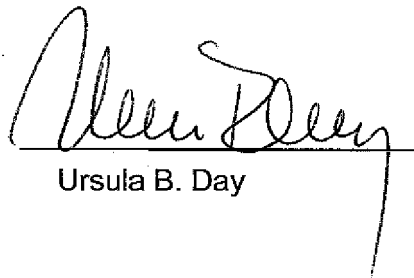


## VERIFICATION OF A TRANSLATION

I, URSULA B. DAY, having a place of business at 708 Third Avenue, Suite 1501, New York, N. Y. 10017, depose and state that:

1. I am familiar with the English and German languages.
2. I have read the attached German language patent application No. 102 59 374.4.
3. The hereto attached English language text is an accurate translation thereof.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code and that such wilful false statements may jeopardize the validity of the application or any patent issuing thereon.



Ursula B. Day

Date: March 11, 2011

# FEDERAL REPUBLIC OF GERMANY

## Priority Certificate Regarding the Filing of a Patent Application

File Number:	102 59 374.4
Date of Filing:	18 December 2002
Applicant/Owner:	ATTO-TEC GmbH, Siegen/DE
Title	Carboxamide-substituted dyes for analytical applications
IPC:	C 09 B 62/78

The attached pieces are a true and accurate copy of the original documents of this patent application.

[SEAL]

Munich, 12 December 2003  
German Patent and Trademark  
Office  
The Commissioner  
On behalf of

# CARBOXAMIDE-SUBSTITUTED DYES FOR ANALYTICAL APPLICATIONS

## Description

The present invention relates to carboxamide-substituted dyes, the production and use of such dyes as labeling groups in analytics.

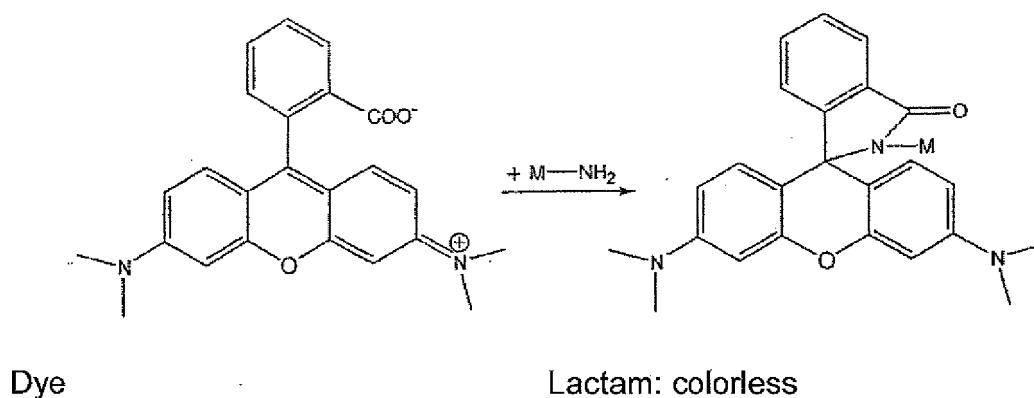
Owing to their very good spectral properties, dyes of the class of xanthene dyes and triphenylmethane dyes and also related derivatives are the preferred labeling groups used in chemical, biological and medical analytics (J. Slavik, *Fluorescent Probes in Cellular and Molecular Biology*, CRC Press, Boca Raton, Ann Arbor, London, Tokyo, 1994). WO 00/64986 and WO 00/64987 describe dyes of the related classes of carbopyronines and amidopyrylium dyes. In this connection, dyes having a very high fluorescence quantum yield are especially important, since fluorescence enables the labeled analyte to be detected with very high sensitivity. However, nonfluorescent derivatives also gain increasing importance as quenchers in special processes.

The use as labeling groups in processes for detecting analytes requires, aside from simple and reliable detectability, good solubility in various solvents, in particular in aqueous systems. Furthermore, compounds of this kind should be simple and inexpensive to produce and have high stability and good storability.

Many dyes of the abovementioned classes of dye possess a carboxyl group which causes the formation of a colorless lactone, owing to its position and the molecular structure as a function of the solvent environment and the pH, for example even in a neutral or weakly basic solution. In this context, cf. K.H. Drexhage, *Structure and Properties of Laser Dyes*, in: F.P. Schäfer, *Topics in Applied Physics*, Vol. 1, *Dye Lasers*, Springer-Verlag, Berlin, Heidelberg, New York, 1973, for example.

The usual covalent coupling, for example via an active ester, of a carboxyl group of this type to the primary amino group of an analyte (peptide etc.) produces an acid

amide which rearranges immediately according to the following diagram to give a lactam:

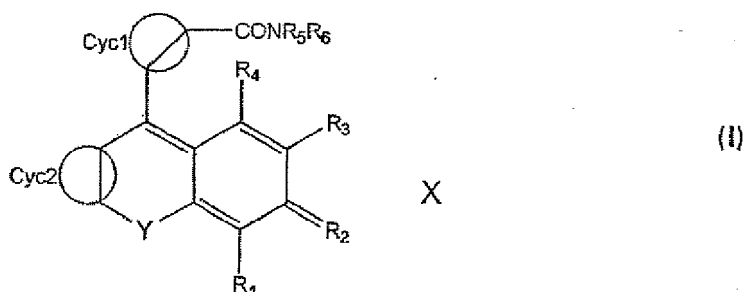


Said lactam, however, is colorless under physiological conditions, thus rendering labeling with the dye unsuccessful, i.e. the labeled analyte cannot be detected by way of absorption and/or fluorescence of the labeling group. Therefore, many known and readily accessible dyes (e.g. rhodamines) are ruled out regarding the use as marker dyes. Previously, this difficulty has been avoided only by introducing additional coupling groups into the dye molecule. However, the precursors required for this are usually difficult to access and require complicated synthesis steps.

WO 02/055512 discloses the preparation of amide derivatives from fluorescein dyes, which comprises first converting the carboxylic acid group at elevated temperatures to give an active ester and then reacting said active ester with a secondary amine in an aqueous solvent mixture. The process according to WO 02/055512 is only applicable to fluorescein and its derivatives and cannot be transferred to other classes of dye. The disclosed process conditions such as temperature and solvents used are in particular not applicable to amino or/and imino group-containing compounds such as, for example, rhodamines. Firstly, the temperatures set for active ester formation result in by- and degradation products, and secondly, the water used as solvent in the reaction of the active ester with an amine can react with said active ester and this in turn results in undesired products.

It is therefore an object of the present invention to modify in a simple manner lactone- or lactam-forming dyes with different functional groups in such a way that they may be used as markers in various fields.

This object is achieved by providing a carboxamide-substituted dye of the formula (I)

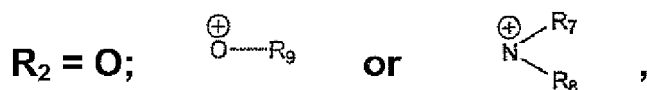


in which

Y = oxygen, sulfur, selenium,  $\text{CR}_a\text{R}_b$ ,  $\text{NR}_c$ , a direct linkage or is  $-\text{R}_{14}$  and  $-\text{R}_{15}$ ;

$\text{R}_1$ ,  $\text{R}_3$ ,  $\text{R}_4$  are independently hydrogen, halogen (such as fluorine, chlorine, bromine, iodine),  $-\text{O}^\ominus$ , a hydroxyl group, thiol group, amino group, ammonium group, sulfo group, phospho group, nitro group, carbonyl group (e.g. keto- or aldehyde group), carboxyl group, a carboxylic acid derivative (such as carboxylate, ester, halide, amide, anhydride), a nitrile group, isonitrile group, cyanate group, isocyanate group, thiocyanate group, isothiocyanate group or a straight-chain, branched or cyclic saturated or unsaturated hydrocarbon moiety having up to 40 carbon atoms;

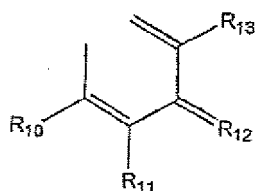
$\text{R}_a$ ,  $\text{R}_b$ ,  $\text{R}_c$  and  $\text{R}_{14}$ ,  $\text{R}_{15}$  independently are as defined for  $\text{R}_1$ ,  $\text{R}_3$ ,  $\text{R}_4$ ;



in which

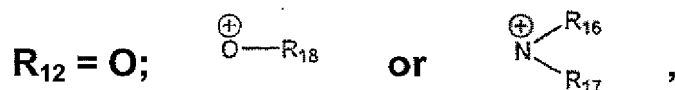
$\text{R}_7$ ,  $\text{R}_8$ ,  $\text{R}_9$  independently are hydrogen or a straight-chain, branched or cyclic saturated or unsaturated hydrocarbon moiety having up to 40 carbon atoms; or

R<sub>1</sub> together with R<sub>2</sub> is



in which

R<sub>10</sub>, R<sub>11</sub>, R<sub>13</sub> are as defined for R<sub>1</sub>, R<sub>3</sub>, R<sub>4</sub>;



in which

R<sub>16</sub>, R<sub>17</sub>, R<sub>18</sub> are as defined for R<sub>7</sub>, R<sub>8</sub>, R<sub>9</sub>;

R<sub>5</sub>, R<sub>6</sub>, independently are a straight-chain, branched or cyclic saturated or unsaturated hydrocarbon moiety having up to 40 carbon atoms;

Cyc1 is an organic moiety which comprises a ring system selected from aromatic, heteroaromatic, quinoidal and cycloaliphatic rings;

Cyc2 is an organic moiety which comprises a ring system selected from aromatic, heteroaromatic, quinoidal and cycloaliphatic rings;

each of said moieties in the compound according to formula (I) being able to form a ring system with one or more neighboring moieties;

and X being one or more mono- or multivalent anions, when required for balancing the charge;

with the proviso that

- Y = oxygen,
- Cyc1 = phenyl or substituted phenyl,
- Cyc2 = hydroxyl-, ether- or ester-substituted phenyl

and


- $R_2 = O$

do not appear in the formula (I) at the same time.

Preference is given to choosing the substituents in the formula (I) in such a way that

- Y = oxygen,
- Cyc1 = phenyl or substituted phenyl,
- Cyc2 = phenyl or substituted phenyl

and

- $R_2 = O$  or  ,

when  $R_7$  or/and  $R_8$  do not form a ring system with neighboring substituents,  
do not appear at the same time.

Surprisingly, it was possible to provide differently functionalized dyes according to the formula (I) which have very good spectral properties such as position of the absorption and fluorescence bands, high extinction coefficients and high fluorescence quantum yields and stabilities. The disadvantage of lactone or lactam formation, which occurs in the case of customarily used carboxyl-substituted dyes, is prevented by the generation of secondary amide groups.

Moreover, the properties of the dye were shown to be controllable by the introduction of various moieties at the amide group. Thus it is possible, for example, to increase the lipophilicity of the dye by introducing long alkyl chains as moieties to the amide group. On the other hand, it is possible to increase the hydrophilicity of the dye by

introducing, for example, sugar residues or other polar groups. This makes it possible to modulate the properties such as solubility behavior in a simple manner. Furthermore, various linkers may be incorporated at the amide group in order to be able to couple or conjugate the dye via said linkers, for example to an analyte to be detected, such as a peptide or the like.

The hydrocarbon groups comprise according to the invention alkyl groups, alkenyl groups, alkynyl groups, cycloalkyl groups, aryl groups and heteroaryl groups. These groups may contain heteroatoms such as oxygen, sulfur or/and nitrogen. Furthermore, it is possible for other substituents to be bound to said groups, which substituents are preferably selected from among halogen (such as fluorine, chlorine, bromine, iodine),  $-O^{\ominus}$ , a hydroxyl group, thiol group, amino group, ammonium group, sulfo group, phospho group, nitro group, carbonyl group (e.g. keto- or aldehyde group), carboxyl group, a carboxylic acid derivative (such as carboxylate, ester, carboxylic acid halide, amide, anhydride), a nitrile group, isonitrile group, cyanate group, isocyanate group, thiocyanate group, isothiocyanate group, imino group, iminium group, alkoxy group, ether group, thioether group and straight-chain, branched or cyclic saturated or unsaturated hydrocarbon groups having up to 40 carbon atoms.

The term "alkyl groups" here comprises straight-chain or branched  $C_1$ - $C_{40}$ -alkyl groups, preferably  $C_2$ - $C_{35}$ -alkyl groups, more preferably  $C_5$ - $C_{30}$ -alkyl groups, still more preferably  $C_8$ - $C_{20}$ -alkyl groups. The alkyl groups are selected, for example, from among methyl, ethyl and straight-chain or branched propyl, butyl, hexyl, decyl, dodecyl and octadecyl groups.

The "alkenyl groups" comprise straight-chain or branched  $C_2$ - $C_{40}$ -alkenyl groups having one or more double bonds at a random position of the hydrocarbon moiety. Depending on the chain length, preference is given to 1-10, 2-8 or 4-6 double bonds per moiety. The alkenyl moieties have 2-40, preferably 4-35, more preferably 8-25, still more preferably 15-20, carbon atoms. Examples of suitable alkenyl groups are



ethenyl, propenyl, butenyl, hexenyl, decenyl, dodecenyl and octadecenyl.

Accordingly, the straight-chain or branched alkynyl moieties contain one or more triple bonds at a random position of the hydrocarbon moiety. Depending on the chain length, preference is given to 1-10, 2-8 or 4-6 triple bonds per moieties. The alkynyl moieties have 2-40, preferably 4-35, more preferably 8-25, still more preferably 15-20, carbon atoms. Examples of suitable alkynyl groups are ethynyl, propynyl, butynyl, hexynyl, decynyl, dodecynyl and octadecynyl.

The cycloalkyl groups may be saturated or unsaturated, i.e. they may optionally have one or more double or/and triple bonds. Preference is given to C<sub>3</sub>-C<sub>40</sub>-cycloalkyl groups, more preferably C<sub>3</sub>-C<sub>20</sub>-cycloalkyl groups, still more preferably C<sub>4</sub>-C<sub>12</sub>-cycloalkyl groups, still more preferably C<sub>5</sub>- or C<sub>6</sub>-cycloalkyl groups. Suitable cycloalkyl groups are in particular selected from among cyclopropyl, cyclobutyl, cyclopentyl, cyclopentenyl, cyclohexyl, cyclohexenyl, cyclohexadienyl and cyclooctyl. Furthermore, the C<sub>3</sub>-C<sub>40</sub>-cycloalkyl groups may be bridged and form bicyclic or polycyclic compounds. Examples of polycyclic moieties are norbornane, norbornene and bicyclo[3.2.2]octyl moieties and the particular substituted derivatives thereof. The cycloalkyl groups moreover comprise aliphatic heterocycles such as, for example, tetrahydropyrrole groups, piperidine groups, dioxane groups, tetrahydrofuran groups. Preferred aryl moieties are C<sub>3</sub>-C<sub>40</sub>-aryl groups, more preferably C<sub>6</sub>-C<sub>20</sub>-aryl groups and C<sub>10</sub>-C<sub>14</sub>-aryl groups. Examples of suitable aryl groups are phenyl groups, naphthyl groups, anthracene groups and phenanthrene groups.

The heteroaryl groups are aromatic moieties having 3-40 carbon atoms and one or more heteroatoms, the latter being selected from among oxygen, sulfur and nitrogen. The heteroaryl groups preferably comprise 1-10, preferably 2-6, more preferably 3-5 heteroatoms. The heteroaryl groups contain 3-40, in particular 3-18, more preferably 4-14, still more preferably 5-8, carbon atoms. Examples of suitable heteroaryl groups are pyrrole groups, pyridine groups, pyrimidine groups, indole groups, furan groups, thiophene groups and thiazole groups.

Examples of hydrocarbon groups containing heteroatoms are heteroatom-containing alkyl groups, for example straight-chain or branched C<sub>1</sub>-C<sub>40</sub>-alkoxy groups, preferably C<sub>1</sub>-C<sub>18</sub>-alkoxy groups, more preferably C<sub>2</sub>-C<sub>14</sub>-alkoxy groups, still more preferably C<sub>6</sub>-C<sub>12</sub>-alkoxy groups. Examples of suitable alkoxy groups are methoxy, ethoxy, straight-chain or branched propoxy, butoxy, decoxy and undecoxy.

Examples of heteroatom-containing aryl groups are aryloxy groups, such as, for example, phenoxy and naphthoxy.

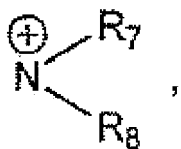
Heteroatom-containing hydrocarbon moieties according to the present invention comprise furthermore, for example, carbonyl groups (such as keto- or aldehyde groups), carboxylic acids, carboxylic acid derivatives (such as halides, esters, amides, anhydrides, carboxylates), ethers, thioethers and alkoxycarbonyl groups, which correspondingly contain 1-40 carbon atoms, preferably 2-35, more preferably 5-30 and also preferably 8-20, carbon atoms.

According to the invention, it is also possible for Cyc1 and Cyc2 to be additionally substituted, the substituents being preferably selected from among halogen (such as fluorine, chlorine, bromine, iodine), -O<sup>⊖</sup>, a hydroxyl group, thiol group, amino group, ammonium group, sulfo group, phospho group, nitro group, carbonyl group (e.g. keto- or aldehyde group), carboxyl group, a carboxylic acid derivative (such as carboxylate, ester, halide, amide, anhydride), a nitrile group, isonitrile group, cyanate group, thiocyanate group, isocyanate group, isothiocyanate group, imino group, iminium group or a straight-chain, branched or cyclic unsaturated or saturated hydrocarbon group as defined above, with optionally one or more heteroatoms.

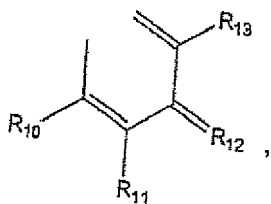
Each of the moieties present in the inventive carboxamide-substituted dye of the formula (I) may, optionally form a ring system together with one or more neighboring moieties. Said ring system preferably comprises 5- or/and 6-membered rings. Preference is given to such a ring system being formed by R<sub>1</sub> with R<sub>8</sub> or/and R<sub>3</sub> with R<sub>7</sub> or R<sub>11</sub> with R<sub>17</sub> or/and R<sub>13</sub> with R<sub>16</sub>.

The anions which may be required for balancing the charge may be selected from inorganic or/and organic anions such as, for example, halides, sulfates, carbonates, phosphates, sulfites, sulfides, hydroxides, alkoxides, carboxylates, nitrates, nitrites, etc.

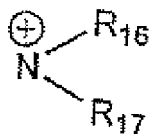
The carboxamide-substituted dyes of the formula (I) are preferably dyes in which Cyc2 in the formula (I) is a nitrogen-containing heterocycle or a ring system which is substituted with at least one amino group. Further preference is given here to R<sub>2</sub> being



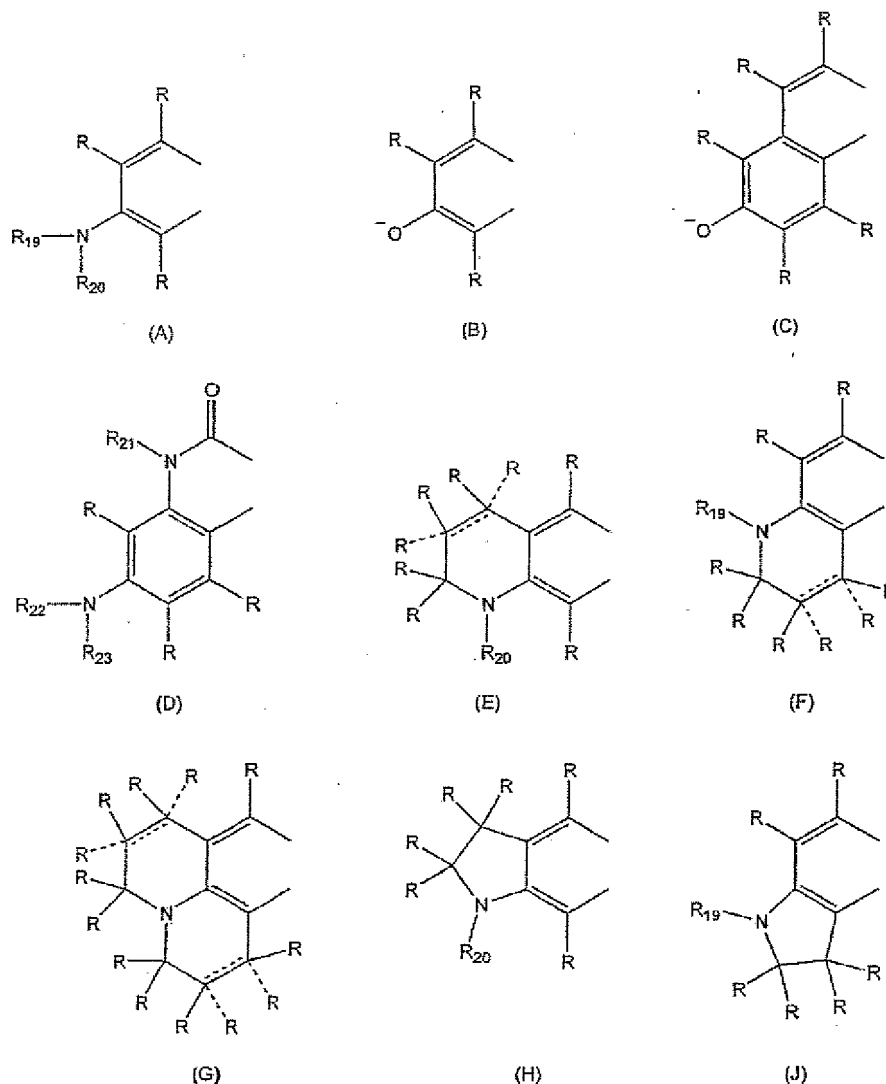
or to R<sub>2</sub> forming, together with R<sub>1</sub>,



with particular preference being given to R<sub>12</sub> being



In a preferred embodiment of the present invention, Cyc2 in the formula (I) has any of the structures (A), (B), (C), (D), (E), (F), (G), (H) or (J):



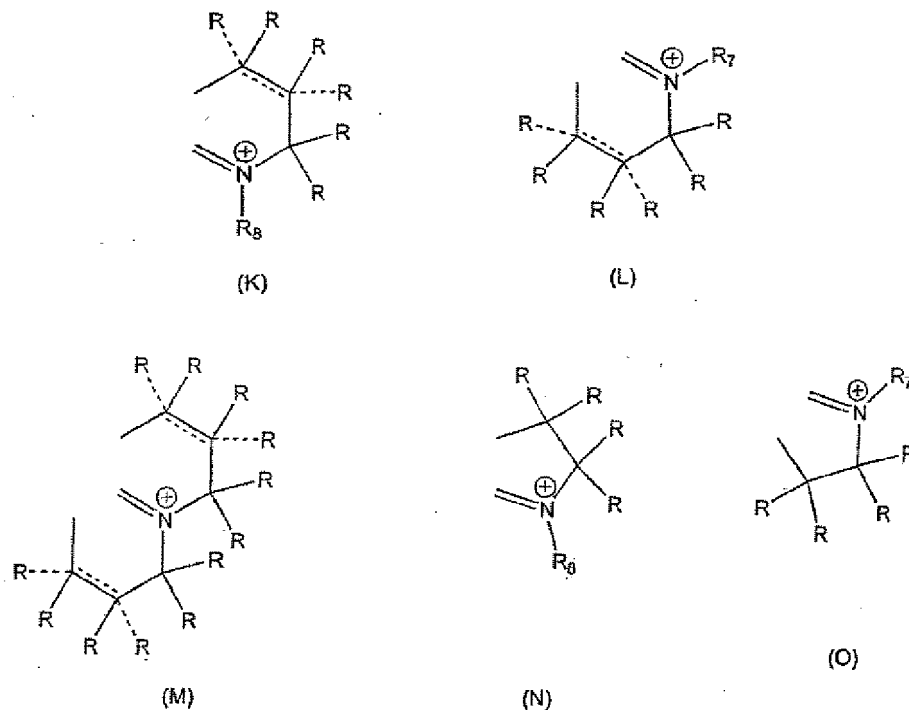
in which R in each case independently is defined as R<sub>1</sub>, R<sub>3</sub>, R<sub>4</sub>; R<sub>19</sub>, R<sub>20</sub> and R<sub>22</sub>, R<sub>23</sub> are independently defined as R<sub>7</sub>, R<sub>8</sub>; and

R<sub>21</sub> is defined as R<sub>7</sub> and the dashed lines are optionally double bonds in the presence of which the moieties bound via a dashed line are absent. The substructures (A) to (J) are fused to the remaining ring structure of the formula (I) in such a way that the linkage sites are connected to one another via single or double bonds. Preference is given to double bonds being located between the linkage sites.

In the structures (A) to (J), neighboring substituents may optionally form further ring systems as defined above. Preference is given here to ring systems which comprise 5- or/and 6-membered rings which may optionally contain further heteroatoms, being

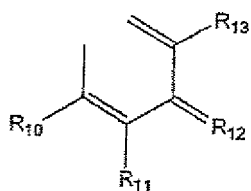
formed by the moieties  $R_{19}$ ,  $R_{20}$  and  $R_{22}$ ,  $R_{23}$  with the in each case neighboring moieties  $R$ . Particular preference is given to Cyc2 having any of the structures (A), (D), (E), (F), (G), (H) or (J).

The ring systems formed starting from  $R_7$  or  $R_8$  together with neighboring substituents preferably result in the following systems (K), (L), (M), (N) or (O):

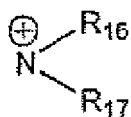


in which  $R$  in each case independently is defined as  $R_1$ ,  $R_3$ ,  $R_4$ , and the dashed lines are optionally double bonds, in the presence of which the moieties bound via a dashed line are absent. In the substructures (K) to (O) too, neighboring substituents may form further ring systems as defined above.

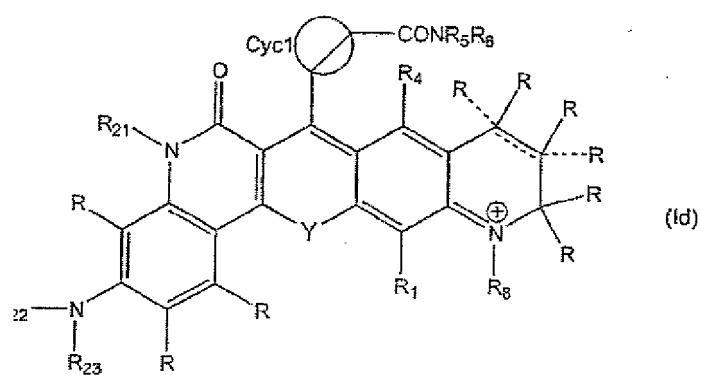
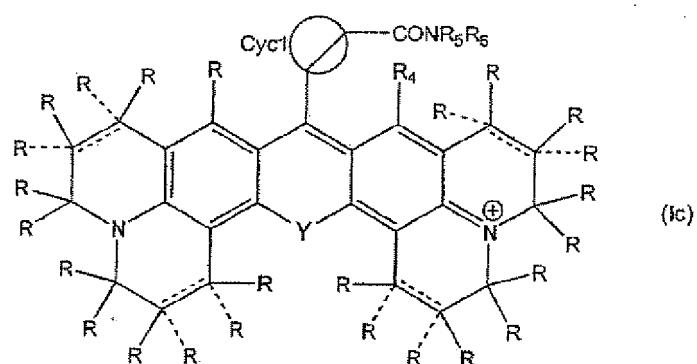
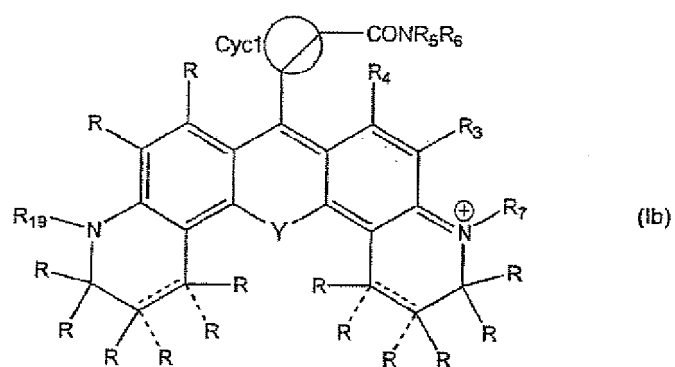
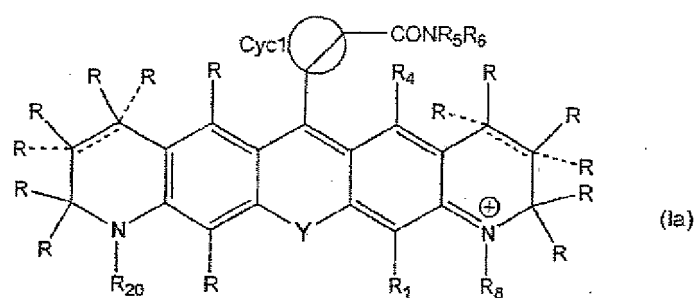
In a further preferred embodiment of the present invention  $R_2$  in the formula (I) together with  $R_1$  is

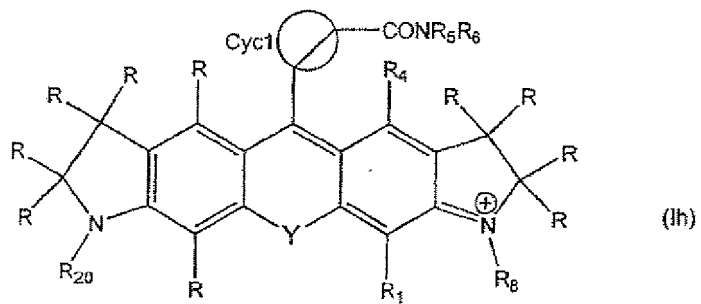
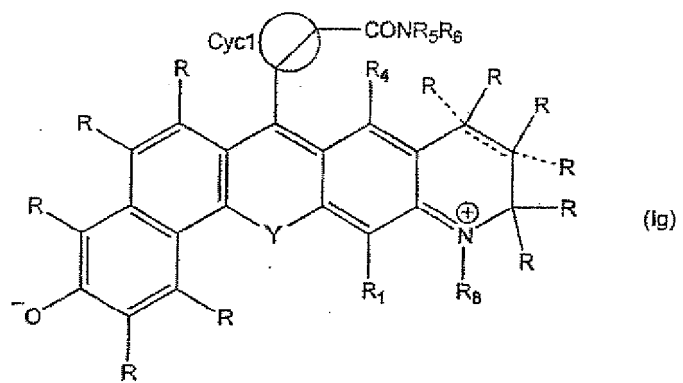
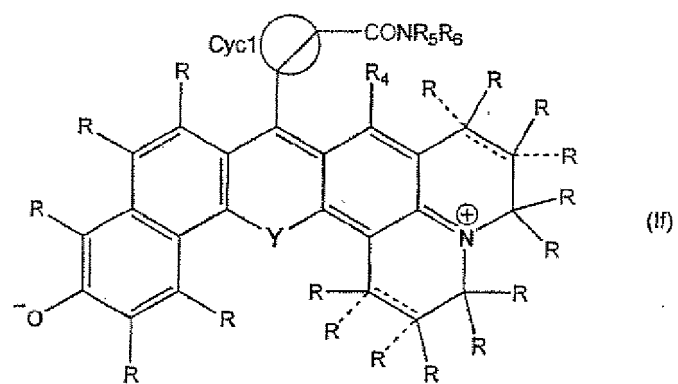
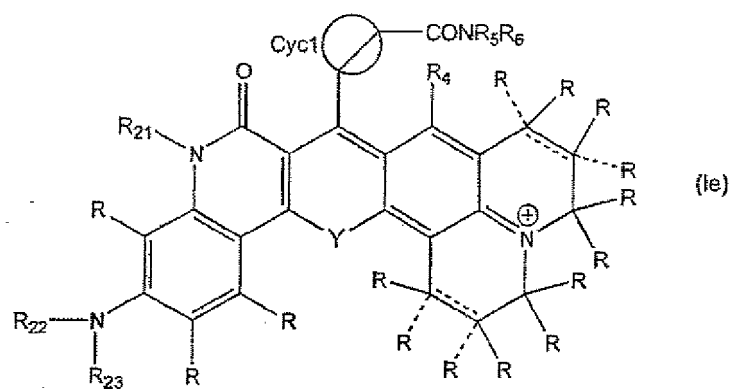


Particular preference is given here to the group of dyes in which  $R_{12}$  is oxygen or



Preferred compound classes of the present invention according to formula (I) are depicted by way of the formulae (Ia) to (Ih):







with the moieties being defined as above.

In the compounds of the invention, Cyc1 is optionally a substituted organic moiety which comprises a ring system preferably selected from among substituted and unsubstituted phenyl, naphthyl, pyridyl and cyclohexyl.

In the carboxamide-substituted dyes of the formula (I) of the present invention, the moieties  $R_5$  or/and  $R_6$  of the carboxamide group preferably have a substituent suitable for coupling to other molecules. Particular preference is given to the possibility of achieving a covalent coupling via such a substituent. Suitable substituents on  $R_5$  or/and  $R_6$  are a carboxyl group, amino group, hydroxyl group, thiol group, cyano group, halogen group or/and groups having unsaturated units such as, for example, double and triple bonds. It is possible, by means of this functionality, to bind the dyes of the invention to biomolecules or supports, for example.

In a preferred embodiment, at least one of the moieties  $R_5$  and  $R_6$  is a carboxy-substituted alkyl group, the moiety which is not substituted with a carboxyl group being preferably an alkyl group.

It is moreover possible, by varying the moieties in the inventive carboxamide-substituted dyes of the formula (I), in particular the readily introducible moieties  $R_5$  or/and  $R_6$ , to adjust and modify in a simple manner the properties of the resulting dye molecules with regard to solubility and fluorescence properties.

If  $R_5$  or/and  $R_6$  carry relatively long alkyl chains, for example (e.g. table 2, compounds 56 and 57), then the lipophilic character increases and the compound is soluble in unpolar media and membranes and may thus be employed, for example, for detecting membrane properties or for measuring molecular distances.

The water solubility of a dye can be improved, for example, when  $R_5$  or/and  $R_6$  carry, for example, sulfonic or phosphonic acid groups (e.g. table 2, compound 55) or have polyether chains. The latter improve the solubility of the compound also in many

organic solvents. Examples of a type of special polyethers are the crown ethers which are used for the fluorescence-sensitive detection of cations and which may also be coupled as aza derivatives to dye molecules by the carboxamide method (e.g. table 2, compound 53).

It is furthermore possible to introduce, for example, sugar residues as  $R_5$  or/and  $R_6$ , thereby making it possible to achieve high solubility in water (see, for example, table 2, compound 51 NK67).

The dyes of the invention moreover achieve the advantage of different meanings of Y enabling the properties of the carboxamide-substituted dyes to be varied, depending on the use purpose. Thus, for example, compounds with Y = selenium have the property of quenching fluorescence. Compounds of this kind are valuable, inter alia, as fluorescence quenchers in bioanalytics.

In contrast, compounds with  $Y = CR_aR_b$  result, for example, in a shift to longer wavelengths in the red range, compared to fluorescein or corresponding xanthene dyes such as rhodamines. Such compounds are useful, inter alia, in order to avoid strong background noise during detection, as is produced by customarily used dye derivatives. Examples of such compounds of the invention are the compounds 13 and 14 of table 1 (NK65, NK66).

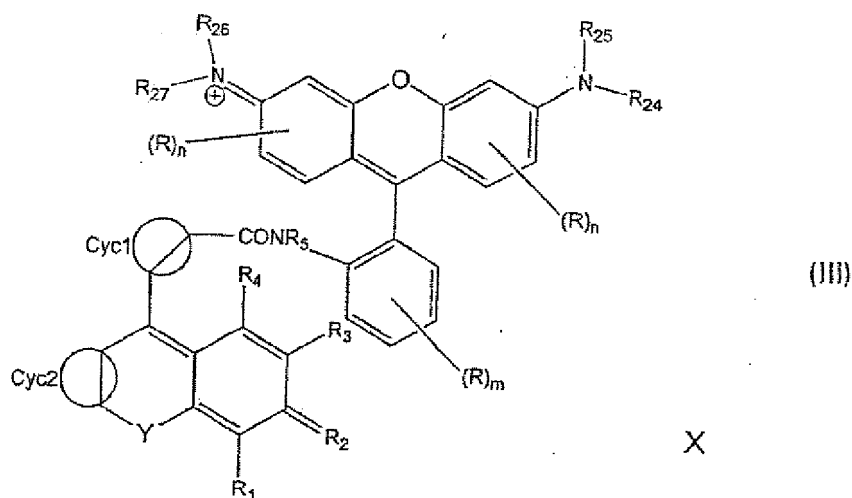
Dyes which correspond to the formula (I), where  $Y = -R_{14}$  and  $-R_{15}$ , are likewise useful fluorescence quenchers in analytics. Examples of such carboxamide-substituted triphenylmethane dyes are the compounds of the invention 29 and 30 in table 1 (NK88, NK89).

Another aspect of the present invention is a multichromophore system in which a carboxamide-substituted dye according to the formula (I) is coupled via  $R_5$  or/ and  $R_6$  to one or more further dye molecules.

The multichromophore system according to the invention is preferably a system in which a further dye molecule according to the formula (I) is coupled to a carboxamide-substituted dye of the invention. An example of a bichromophore system of this kind is compound 59 in table 3 (NK69).

Preference is given to the coupling to the further carboxamide-substituted dye(s) being carried out in each case via the moiety  $R_5$  or/and  $R_6$ . For example, the nitrogen atoms of the carboxamide group in the dye units are linked via alkyl chains.

A further preferred embodiment of the multichromophore system according to the invention is a bichromophore system of the formula (III).



where the moieties are as defined above and  $R_{24}$ ,  $R_{25}$  and  $R_{26}$ ,  $R_{27}$  are defined as  $R_7$ ,  $R_8$ , with  $n$  independently being 0, 1, 2 or 3 and  $m$  being 0, 1, 2, 3 or 4.

An energy transfer (FRET) between the dye molecules takes place in the multichromophore, in particular bichromophore, systems according to the present invention, thereby enabling particular spectroscopic properties to be achieved.

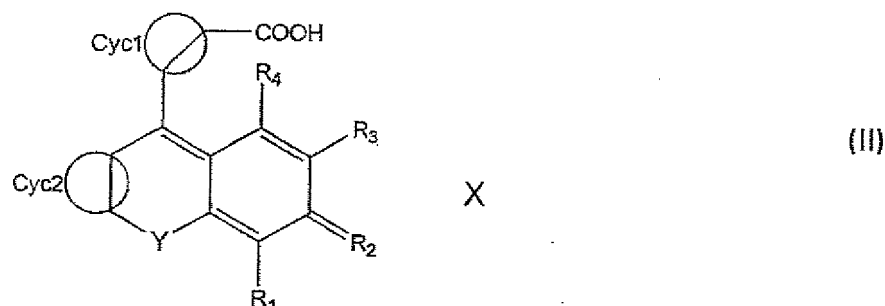
The carboxamide-substituted dyes of the invention distinguish themselves in

particular by not displaying the formation of lactones or lactams which occurs in the case of conventional carboxyl group-substituted dyes. The introduction of the carboxamide group also enables the dye molecules to be functionalized in a variable manner, and the latter thus have excellent suitability for a wide variety of applications such as analytical processes. Surprisingly, introduction of the carboxamide group does not substantially alter the very good spectral properties of the starting dyes, i.e. the absorption and fluorescence bands, high extinction coefficients and high fluorescence quantum yields and stability. It was merely observed that the absorption and fluorescence maxima of some dyes undergo a shift to longer wavelengths by 10 nm on average.

It was furthermore found in the context of the present invention that a dye having an appropriate carboxyl group with a tendency to form lactones or lactams (formula (II)) reacts to give the desired inventive carboxamide-substituted dye of the formula (I) by simple reaction of an activated derivative of said dye, previously formed from said carboxyl group, with a secondary amine in common solvents such as acetonitrile or DMF. It is astonishing here that the amide is produced with good yields, even with varying steric and electronic states in the dye precursor. In particular, carboxyl group-containing dyes which differ substantially from fluorescein derivatives regarding the electronic states in the dye molecule were not expected, but were nevertheless able to be successfully converted to the carboxamide-substituted dyes of the invention.

The present invention therefore further relates to a process for preparing carboxamide-substituted dyes of the formula (I), comprising the following steps:

- (a) converting the carboxyl group of a dye of the formula (II)



- in which the moieties are defined as indicated above, into an activated form;
- (b) reacting the activated dye obtained in step (a) with a secondary amine  $\text{HNR}_5\text{R}_6$ ; and
- (c) optionally isolating the carboxamide-substituted dye of the formula (I) obtained in step (b).

A carboxyl-containing dye according to the formula (II) is preferably activated by converting the carboxyl group into an active ester or an acid chloride. The activation is carried out using common processes well known to the skilled person. In general, the active esters are prepared using customary reagents such as, for example, N-hydroxysuccinimide, N-hydroxyphthalimide, N-hydroxynaphthalimide, O-(N-succinimidyl)-N,N,N',N'-tetramethyluronim tetrafluoroborate (TSTU). Work is preferably carried out at temperatures of from  $0^\circ\text{C}$  to about  $60^\circ\text{C}$ , more preferably at  $10^\circ\text{C}$  to  $40^\circ\text{C}$ , most preferably at  $20^\circ\text{C}$  to  $30^\circ\text{C}$ , in particular at room temperature.

In particular, activated dyes derived from rhodamine are prepared at room temperature in order to avoid decomposition of the rhodamine derivatives. The reaction times vary in this case, depending on the structure of the dye, but the reaction is generally complete after about 5-48 hours, preferably 8-24 hours.

The activated dye obtained in step (a) is reacted with secondary amines which contain the moieties  $\text{R}_5$  and  $\text{R}_6$  desired later in the carboxamide-substituted dye of the formula (I). The reaction is preferably carried out at room temperature up to temperatures of approximately  $100^\circ\text{C}$ , preferably at temperatures of from about  $50^\circ\text{C}$

to 90°C, more preferably approximately 60°C to 80°C. For this purpose, the active ester and the appropriate secondary amine are dissolved in an aprotic solvent such as, for example, acetonitrile, DMF, DMSO etc. and heated (carboxamide process). During synthesis of the carboxamide-substituted dyes derived from rhodamine, which are preferred according to the invention, particular care must be taken that the solvent is anhydrous in order to avoid a reaction with the active ester.

Amide formation is usually complete after several hours. The carboxamide-substituted dye obtained in step (b) is optionally isolated from the reaction mixture by processes well known to the skilled person, such as, for example, extracting, filtering, work-up by column chromatography, distillation etc.

A substantial advantage of the process described is the unlimited applicability to all dyes of the formula (II) which contain in particular a carboxyl group which is in the ortho position or, owing to its position, tends to form lactones. Examples of classes of dye which may be employed are listed in figure 1. Especially remarkable here is the fact that it is also possible to successfully convert amino and imino or iminium group-containing dyes such as, for example, rhodamines by means of the process of the invention.

The resulting dyes of the invention are particularly well suited as labeling groups in analytical processes.

The present invention therefore further relates to the use of an inventive carboxamide dye according to the formula (I) for the qualitative or/and quantitative determination of an analyte. The dyes of the invention exhibit very good results, both in chemical and in medical and biological detection processes. The determination may be carried out, for example, in aqueous liquids such as body fluids, for example blood, serum, plasma or urine, wastewater samples or food, using detection processes known to the skilled person. In this context, the process may be carried out both as wet assay, for example in a cuvette, or as dry assay on an appropriate reagent support.

Determination of the analytes may be carried out via single reaction or by way of a reaction sequence.

In order to detect an analyte, the carboxamide-substituted dye of the invention is preferably coupled to said analyte or/and a support. In a particularly preferred embodiment of the present invention, the dye is coupled to a component of a detection reagent. Such a component is preferably a universal receptor or an analyte analog. Examples of binding partners of this kind are preferably selected from among peptides, polypeptides, nucleic acids, nucleosides, nucleotides, nucleic acid analogs and haptens.

The detection comprises in particular immunological detection or/and detection by nucleic acid hybridization. The analyte to be detected is preferably selected from among peptides, polypeptides, antibodies, nucleic acids, nucleic acid analogs, haptens, cells, cell components, viruses, viral components, metabolites, hormones, neurotransmitters and medicaments.

Any suitable material may be selected as support, for example porous glass, plastics, ion exchange resins, dextrans, cellulose, cellulose derivatives and hydrophilic polymers.

The present invention further relates to a conjugate of a binding partner with a carboxamide-substituted dye according to formula (I). The binding partner here is preferably selected from among peptides, polypeptides, nucleic acids, nucleosides, nucleotides, nucleic acid analogs and haptens. The conjugates may be used, for example, in nucleic acid hybridization processes or immunochemical processes. Such processes are described, for example, in Sambrook et al., *Molecular Cloning, A Laboratory Manual*, 1989, Cold Spring Harbor.

Coupling to the analyte to be detected or/and to the component of a detection reagent or/and to the support is preferably carried out via the substituents  $R_5$  or/and

$R_6$  of the carboxamide-substituted dye of the formula (I). Particularly preferred is the formation of a covalent bond. This is accomplished in particular by means of the active ester method well known to the skilled person. Particularly suitable moieties  $R_5$  or  $R_6$  for this purpose are carboxyl groups or substituents containing carboxyl groups. Such a carboxyl group is in turn itself activatable and may be used preferably for couplings and conjugate formations of the dye of the invention to analytes, components of detection reagents or supports.

For example, the succinimidyl ester (NHS ester) prepared from the dye NK 50 (compound 1) reacts with benzylamine in acetonitrile to give the corresponding conjugate, with the reaction being complete within 30 minutes, according to HPLC or DC analysis. The reaction with aminoethylmaleimide to give the maleimide derivative is also complete after a few hours. This compound was in turn able to be successfully reacted with N-acetylcysteine. The method described can be used to prepare from the terminal carboxyl group all the common derivatives which are used, for example, for coupling to amino and thiol groups of biomolecules.

Compared with conventional dyes, the carboxamide-substituted dyes of the invention have the advantage that, although they contain an activatable carboxyl group, this group does not have a tendency to form lactones or lactams.

The following figures and examples are intended to illustrate the invention in more detail.

#### Figures

Figure 1 lists representatives of various carboxyl-containing classes of dye, the substituents having been omitted or not being referred to in any detail.

Figure 2 lists carboxamide-substituted dyes of the invention, having on  $R_5$  or  $R_6$  a carboxyl group which can be coupled, with the particular spectral data (absorbance maximum, fluorescence maximum and fluorescence quantum yield).



Figure 3 indicates further examples of carboxamide-substituted dyes of the invention and their spectral data (absorbance maximum, fluorescence maximum and fluorescence quantum yield).

Figure 4 lists bichromophore systems of the invention.

Figure 5 depicts the absorbance and, respectively, fluorescence spectrum of compound NK51 in ethanol.

Figure 6 depicts the absorbance and, respectively, fluorescence spectrum of compound NK56 in ethanol.

Figure 7 depicts the absorbance and, respectively, fluorescence spectrum of compound NK63 in ethanol.

Figure 8 depicts the absorbance and, respectively, fluorescence spectrum of compound NK65 in ethanol.

## Examples

The dyes used for the carboxamide process are either commercially available or to be prepared according to syntheses known from the literature or processes known to the skilled person. The secondary amines used are likewise in most cases commercially available or accessible by methods known from the literature. The preferred process for preparing compounds of the invention is described by way of example on the basis of the structures 1 (NK 50), 4 (NK 56), 51 (NK 67) and 18 (NK 79).

Example 1: Preparation of compounds of the invention

Compound 1 (NK 50)

Stage 1: Rhodamine B NHS ester

1 g (2.1 mmol) of commercial rhodamine B (chloride) and 700 mg (2.4 mmol) of O-(N-succinimidyl-N,N,N',N'-tetramethyluronium tetrafluoroborate (TSTU) are taken up together with 700  $\mu$ l of Hünig base (N-ethyldiisopropylamine) in 50 ml of dry acetonitrile and stirred at room temperature for about two hours and the reaction is monitored by thin-layer chromatography. After the reaction is complete, a little colorless precipitate is removed by filtration and the filtrate is treated with 2 ml of perchloric acid (60% strength). The active ester is precipitated by quickly adding dropwise ice-cold 10% strength sodium perchlorate solution, filtered off with suction, washed with ice water and diethyl ether and finally rigorously dried over phosphorus pentoxide in a desiccator under reduced pressure.

Yield: 0.8 g

ESI mass spectrum:  $m/z$  = 540.2

Stage 2: NK 50

0.5 g (0.78 mmol) of rhodamine B NHS ester (perchlorate) and 0.25 g (1.56 mmol) of 4-methylaminobutyric acid hydrochloride are suspended in 40 ml of acetonitrile, treated with 270  $\mu$ l of Hünig base and heated to reflux. The reaction is monitored by thin-layer chromatography and stopped, after the NHS ester has disappeared, usually after four hours. The reaction mixture is evaporated to dryness and purified by column chromatography on neutral aluminum oxide (activity I). The gradient of the eluent is from ethanol to water. The dye fractions are pooled and concentrated in a rotary evaporator. The residue is dissolved in chloroform and extracted by shaking with water saturated with sodium chloride, in order to remove 4-methylaminobutyric acid residues. The chloroform phase is dried and concentrated in a rotary evaporator.

Yield: 0.3 g

$^1\text{H}$  NMR data in DMSO- $d_6$ :

$\delta$  1.2 (T, 12H,  $-\text{CH}_3$ ); 1.6 (M, 2H,  $-\text{CH}_2-$ ); 2.6 (D, 2H,  $-\text{CH}_2-$ ); 2.8 (S, 3H,  $-\text{CH}_3$ ); 3.1 (D, 2H,  $-\text{CH}_2-$ ); 3.6 (Qu, 8H, N- $\text{CH}_2-$ ); 6.9 (S, 2H, ArH); 7.1 (M, 4H, ArH); 7.6 (M, 4H, ArH)

ESI mass spectrum:  $m/z$  = 542.2

The secondary amide bond formed was shown, on the basis of the dye NK 50 (compound 1), to be stable under the common physiological and experimental conditions. The carboxamide bond is not cleaved at room temperature and a pH of between pH 3 and pH 11 within 24 hours. At an elevated temperature (100°C), cleavage becomes noticeable only in the pH range above pH 11.

#### Compound 4 (NK 56)

##### Stage 1: MR 33 NHS ester

300 mg (0.69 mmol) of MR 33 are dissolved with 157 mg (0.75 mmol) of dicyclohexylcarbodiimide and 87 mg (0.75 mmol) of N-hydroxysuccinimide in 15 ml of acetonitrile and stirred at room temperature for 24 hours. The reaction mixture is filtered and the filtrate is then treated with diethyl ether. The resultant precipitate is filtered off with suction and dried.

Yield: 80 mg

##### Stage 2: NK 56

40 mg (0.075 mmol) of MR 33 NHS ester are dissolved together with 25 mg (0.15 mmol) of 4-methylaminobutyric acid hydrochloride and 30 µl of Hünig base in 20 ml of acetonitrile and heated to reflux for about one hour. The reaction is monitored by thin-layer chromatography. After the reaction is complete, the mixture is concentrated to dryness in a rotary evaporator and the residue is purified by column chromatography on silica gel using a gradient from chloroform over ethanol to water.

#### Compound 51 (NK 67)

0.5 g (0.78 mmol) of rhodamine B NHS ester (perchlorate) and 0.3 g (1.56 mmol) of N-methyl-D-glucamine are dissolved in 20 ml of acetonitrile and heated to reflux. The product is isolated by column chromatography on silica gel with chloroform/ethanol 9:1.

ESI mass spectrum:  $m/z = 620.3$

## Compound 18 (NK 136)

### Stage 1: NK 135

11 g of resorcinol (100 mmol) and 10.8 g of cyclohexanedicarboxylic acid anhydride (isomer mixture, 70 mmol) are mixed, finely ground in a mortar and melted at about 180°C on an oil bath. The melt solidifies during the reaction. The reaction is complete after approx. 4 hours. After cooling, the solid is crushed in a mortar and extracted in boiling water, filtered off with suction and thoroughly washed with water.

The crude product can be used for the next stage without purification. For further purification, the product may be dissolved in diluted sodium hydroxide solution and reprecipitated with semi-concentrated sulfuric acid.

### Stage 2: NK 135 NHS ester

100 mg (0.3 mmol) of NK 135 are dissolved in 10 ml of acetonitrile with addition of about 100 µl of DMF and 0.5 ml of 20% strength aqueous tetraethylammonium hydroxide solution and admixed with 72 mg (0.35 mmol) of dicyclohexylcarbodiimide and 40 mg (0.35 mmol) of N-hydroxysuccinimide. The solution is stirred at room temperature for 12 hours, then concentrated to half the volume at about 40°C under reduced pressure, filtered, and subsequently a solid is precipitated by adding water. The crude product is filtered off with suction, washed with a little water and dried over phosphorus pentoxide under reduced pressure. The solid also contains unreacted reactant.

### Stage 3: NK 136

100 mg of the solid obtained in this way are suspended with 200 mg of 4-methylaminobutyric acid hydrochloride in 10 ml of acetonitrile and heated to reflux with 250 µl of Hünig base for three hours. The reaction solution is concentrated to dryness in a rotary evaporator and isolated by column chromatography on silica gel

using a gradient from chloroform to ethanol. The last colored zone contains the desired product.

ESI mass spectrum:  $m/z = 434.1$

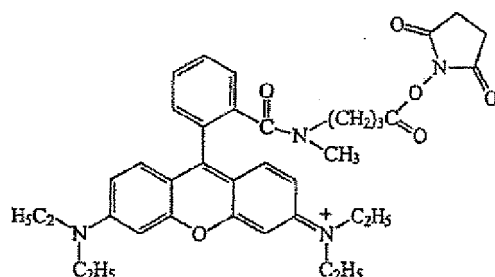
#### Example 2: Examples of conjugate formation

##### NK 50 NHS ester

0.5 g (0.78 mmol) of NK 50 perchlorate are dissolved in 20 ml of dry acetonitrile, admixed with 250 mg (0.85 mmol) of TSTU and 170  $\mu\text{l}$  (1 mmol) of Hünig base and stirred at room temperature. After the reaction is complete, the volume of the solution is reduced to about a quarter under reduced pressure, treated with 1 ml of 60% strength perchloric acid, and the dye is precipitated by adding dropwise 20% sodium perchlorate solution. The solid is filtered off with suction, thoroughly washed with water and diethyl ether and dried over phosphorus pentoxide under reduced pressure using an oil pump.

Yield: 0.4 g

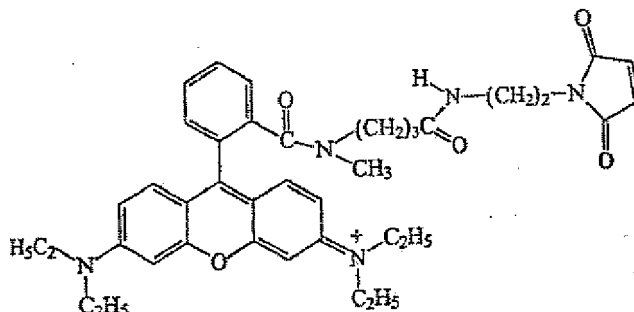
ESI mass spectrum:  $m/z = 639.3$



##### NK 50 aminoethylmaleimide

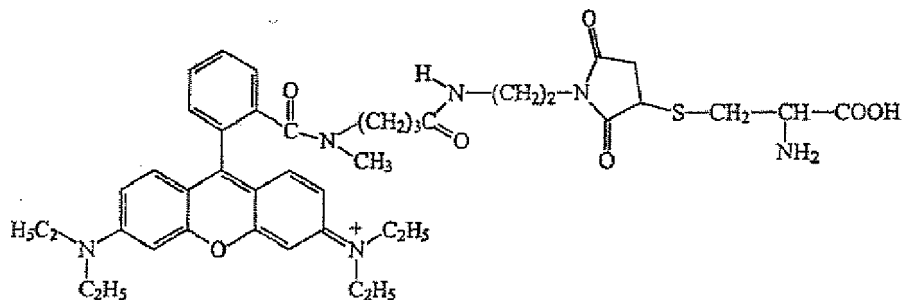
0.1 mmol of NK 50 NHS ester are dissolved in 5 ml of dry acetonitrile, and admixed with 0.15 mmol of aminoethylmaleimide and 30  $\mu\text{l}$  of Hünig base. The solution is stirred at room temperature for 5 hours, filtered and added dropwise to diethyl ether.

The precipitate obtained in this way is dried under reduced pressure using an oil pump.



#### NK 50 aminoethylmaleimide-cysteine conjugate

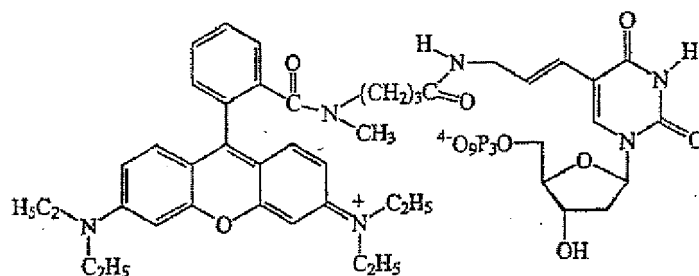
0.1 mmol of NK 50 aminoethylmaleimide are dissolved in 20 ml of ethanol and admixed in portions with 0.15 mmol of cysteine in total. The mixture is stirred at room temperature and, after about 2 hours, approx. 50 ml of a 10% strength sodium perchlorate solution are added dropwise. The precipitated solid is filtered off and dried over phosphorus pentoxide in a desiccator under reduced pressure.



#### NK 50 dUTP conjugate

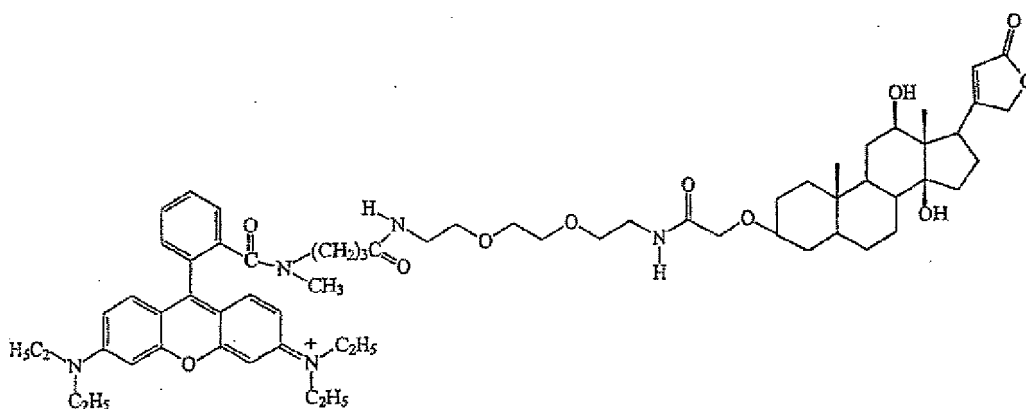
10  $\mu$ mol of 5-(3-aminoallyl)-dUPT are dissolved in 0.5 ml of 0.1 M sodium borate buffer (pH 8) and treated with a solution of 5  $\mu$ mol of NK 50 active ester in 1 ml of

amine-free N,N-dimethylformamide. The solution is stirred at room temperature for 15 hours, the solvent is distilled off under reduced pressure and the residue is purified by chromatography.



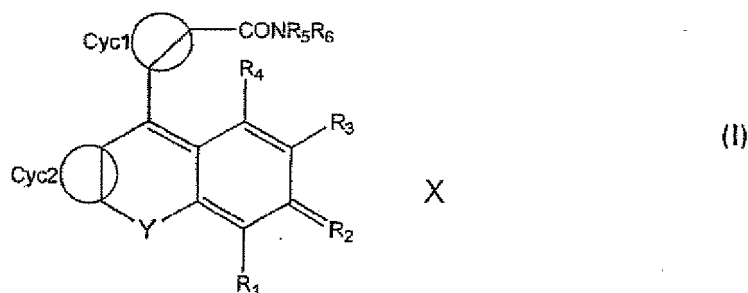
NK 50 digoxin 3-carboxymethyl ether-diaminodioxaoctane conjugate (Dig-CME-DADOO)

0.02 mmol of NK 50 active ester are mixed with 0.02 mmol of Dig-CME-DADOO in acetonitrile with stirring at room temperature for 18 hours. The solution is concentrated and the residue is purified by chromatography.



## Claims

1. A carboxamide-substituted dye of the formula (I)



in which

Y = oxygen, sulfur, selenium,  $\text{CR}_a\text{R}_b$ ,  $\text{NR}_c$ , a direct linkage or is  $-\text{R}_{14}$  and  $-\text{R}_{15}$ ;

$\text{R}_1$ ,  $\text{R}_3$ ,  $\text{R}_4$  are independently hydrogen, halogen,  $-\text{O}^\ominus$ , a hydroxyl group, thiol group, amino group, ammonium group, sulfo group, phospho group, nitro group, carbonyl group, carboxyl group, a carboxylic acid derivative, a nitrile group, isonitrile group, cyanate group, isocyanate group, thiocyanate group, isothiocyanate group or a straight-chain, branched or cyclic saturated or unsaturated hydrocarbon group having up to 40 carbon atoms;

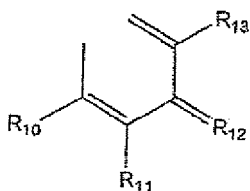
$\text{R}_a$ ,  $\text{R}_b$ ,  $\text{R}_c$  and  $\text{R}_{14}$ ,  $\text{R}_{15}$  independently are as defined for  $\text{R}_1$ ,  $\text{R}_3$ ,  $\text{R}_4$ ;



in which

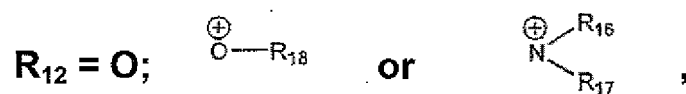
$\text{R}_7$ ,  $\text{R}_8$ ,  $\text{R}_9$  independently are hydrogen or a straight-chain, branched or cyclic saturated or unsaturated hydrocarbon group having up to 40 carbon atoms; or  $\text{R}_1$  together with  $\text{R}_2$  is





in which

$R_{10}$ ,  $R_{11}$ ,  $R_{13}$  are as defined for  $R_1$ ,  $R_3$ ,  $R_4$ ;



in which

$R_{16}$ ,  $R_{17}$ ,  $R_{18}$  are as defined for  $R_7$ ,  $R_8$ ,  $R_9$ ;

$R_5$ ,  $R_6$ , independently are a straight-chain, branched or cyclic saturated or unsaturated hydrocarbon group having up to 40 carbon atoms;

Cyc1 is an organic moiety which comprises a ring system selected from aromatic, heteroaromatic, quinoidal and cycloaliphatic rings;

Cyc2 is an organic moiety which comprises a ring system selected from aromatic, heteroaromatic, quinoidal and cycloaliphatic rings;

each of said moieties in the dye of the formula (I) being able to form a ring system with one or more neighboring moieties;

and X being one or more mono- or multivalent anions, when required for balancing the charge;

with the proviso that

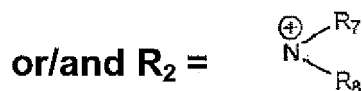
- Y = oxygen,
- Cyc1 = phenyl or substituted phenyl,

- Cyc2 = hydroxyl-, ether- or ester-substituted phenyl  
and

-  $R_2 = O$

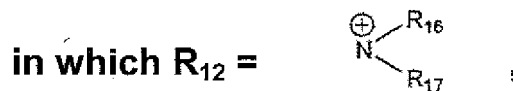
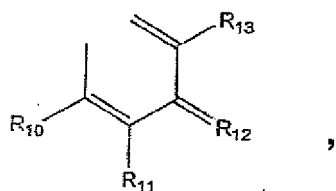
do not appear in the formula (I) at the same time.

2. The carboxamide-substituted dye as claimed in claim 1, in which Cyc2 is a nitrogen-containing heterocycle or a ring system substituted with at least one amino group



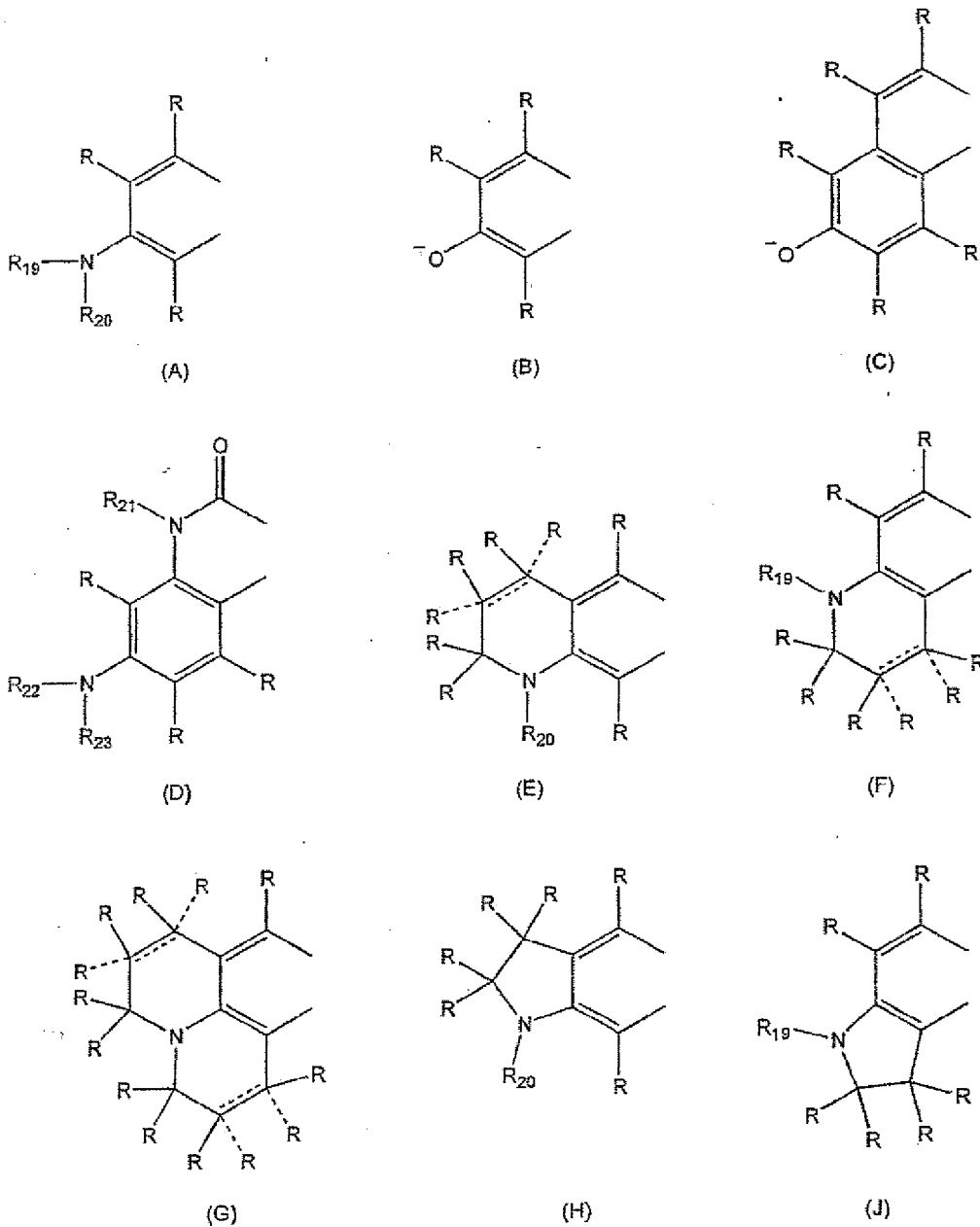
or,

together with  $R_1 =$



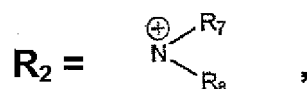
in which  $R_7, R_8, R_{10}, R_{11}, R_{13}$  and  $R_{16}, R_{17}$  are as defined in claim 1.

3. The carboxamide-substituted dye as claimed in claim 1 or 2, in which Cyc2 in the formula (I) has a structure (A), (B), (C), (D), (E), (F), (G), (H) or (J),



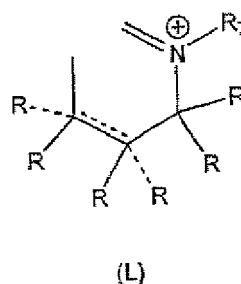
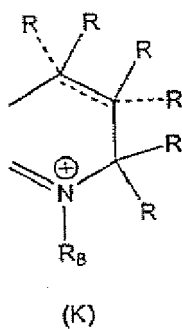
in which R in each case independently is defined as R<sub>1</sub>, R<sub>3</sub>, R<sub>4</sub> in claim 1; R<sub>19</sub>, R<sub>20</sub> and R<sub>22</sub>, R<sub>23</sub> are independently defined as R<sub>7</sub>, R<sub>8</sub> in claim 1; and R<sub>21</sub> is defined as R<sub>7</sub> in claim 1 and the dashed lines are optionally double bonds in the presence of which the moieties bound via a dashed line are absent.

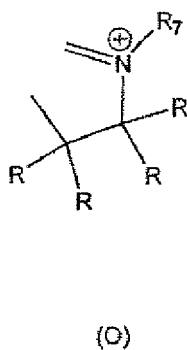
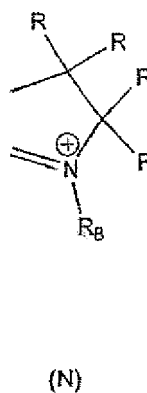
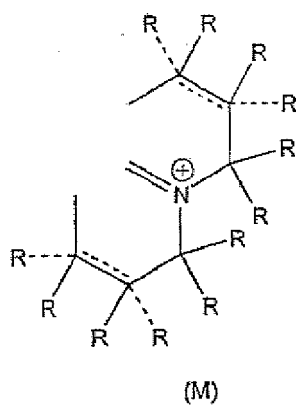
4. The carboxamide-substituted dye as claimed in any of the preceding claims, in which Cyc1 is substituted or unsubstituted phenyl, naphthyl, pyridyl or cyclohexyl.
5. The carboxamide-substituted dye as claimed in any of the preceding claims, in which



where  $R_7$  and  $R_8$  are as defined in claim 1.

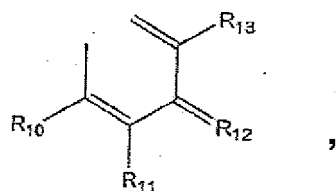
6. The carboxamide-substituted dye as claimed in claim 5, in which  $R_1$  is bridged with  $R_8$  or/and  $R_3$  is bridged with  $R_7$  and forms a ring system.
7. The carboxamide-substituted dye as claimed in claim 6, in which the ring system/s comprise(s) 5- or 6-membered rings.
8. The carboxamide-substituted dye as claimed in claim 7, in which a ring system of the structure (K), (L), (M), (N) or (O) is formed:





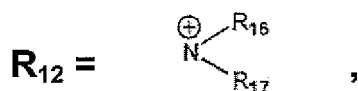
in which R in each case independently is defined as  $R_1$ ,  $R_3$ ,  $R_4$  and  $R_7$ ,  $R_8$  are as defined in claim 1,  
and the dashed lines are optionally in the presence of which the moieties bound via a dashed line are absent.

9. The carboxamide-substituted dye as claimed in any of claims 1 to 4, in which  $R_2$  together with  $R_1$  is



where  $R_{10}$ - $R_{13}$  are as defined in claim 1.

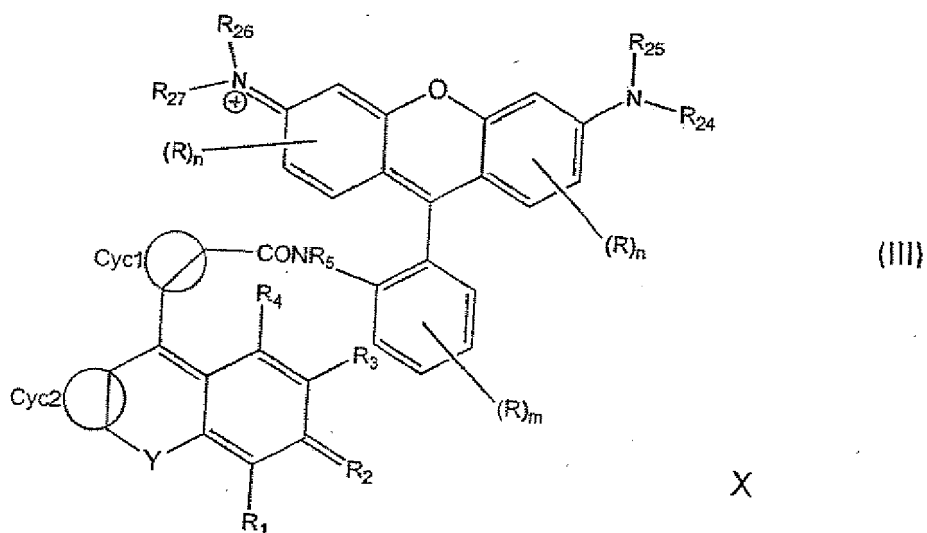
10. The carboxamide-substituted dye as claimed in claim 9, in which  $R_{12} = O$ .
11. The carboxamide-substituted dye as claimed in claim 9, in which



where  $R_{16}$  and  $R_{17}$  are as defined in claim 1.

12. The carboxamide-substituted dye as claimed in any of the preceding claims, in which  $Y = \text{oxygen}$ .
13. The carboxamide-substituted dye as claimed in any of claims 1 to 11, in which  $Y = \text{sulfur, selenium or } CR_aR_b$ ,  $R_a$  and  $R_b$  being as defined in claim 1.
14. The carboxamide-substituted dye as claimed in any of claims 1 to 11, in which  $Y = r$  moieties  $-R_{14}$  and  $-R_{15}$ ,  $R_{14}$  and  $R_{15}$  being as defined in claim 1.
15. The carboxamide-substituted dye as claimed in claim 8, in which  $\text{Cyc1}$  is optionally substituted phenyl,  $\text{Cyc2}$  has the structure (E) and  $Y = \text{oxygen}$  and  $R_7$  and  $R_3$  form a ring system (K),  $R_7$  and  $R_3$  being as defined in claim 1.

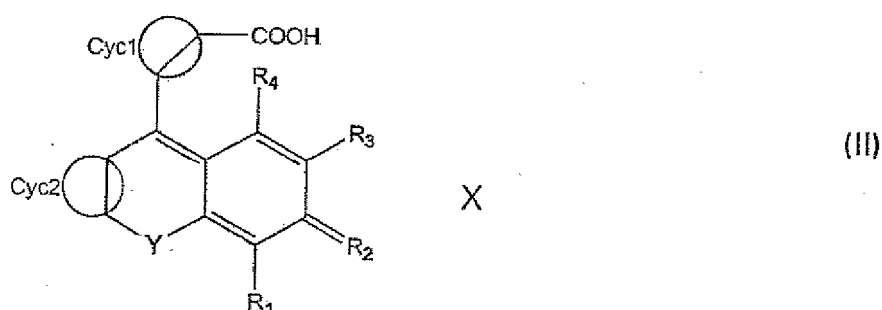
16. The carboxamide-substituted dye as claimed in claim 8, in which Cyc1 is optionally substituted phenyl, Cyc2 has the structure (A) and Y = sulfur, selenium or CR<sub>a</sub>R<sub>b</sub>, R<sub>a</sub> and R<sub>b</sub> being as defined in claim 1.
17. A multichromophore system in which a carboxamide-substituted dye as claimed in any of claims 1 to 16 is coupled via R<sub>5</sub> or/and R<sub>6</sub> to one or more further dye molecules, R<sub>5</sub> and R<sub>6</sub> being as defined in claim 1.
18. The multichromophore system as claimed in claim 17, in which the one or more further dye molecules are carboxamide-substituted dyes as claimed in any of claims 1 to 16.
19. The multichromophore system as claimed in claim 18, in which coupling takes place on R<sub>5</sub> or/and R<sub>6</sub> of the further carboxamide-substituted dyes, R<sub>5</sub> and R<sub>6</sub> being as defined in claim 1.
20. The multichromophore system as claimed in claim 17 of the formula (III)



where the moieties are as defined in claim 1, R in each case independently is defined as R<sub>1</sub>, R<sub>3</sub>, R<sub>4</sub> and R<sub>24</sub>, R<sub>25</sub> and R<sub>26</sub>, R<sub>27</sub> are defined as R<sub>7</sub>, R<sub>8</sub> in claim 1, with n independently being 0, 1, 2 or 3 and m being 0, 1, 2, 3 or 4.

21. A process for preparing carboxamide-substituted dyes of the formula (I) as claimed in any of claims 1 to 16, comprising the following steps:

(a) converting the carboxyl group of a dye of the formula (II)



in which the moieties are defined as indicated in claim 1, into an activated form;

(b) reacting the activated dye obtained in step (a) with a secondary amine HNR<sub>5</sub>R<sub>6</sub>; and

(c) optionally isolating the carboxamide-substituted dye of the formula (I) obtained in step (b).

22. The process as claimed in claim 21, in which step (a) is carried out at temperatures of from room temperature to 60°C.
23. The process as claimed in claim 21 or 22, in which an aprotic solvent is used in step (b).
24. The process as claimed in any of claims 21 to 23, in which N-hydroxysuccinimide, N-hydroxyphthalimide, N-hydroxynaphthalimide, O-(N-succinimidyl)-N,N,N',N'-tetramethyluronim tetrafluoroborate (TSTU) are



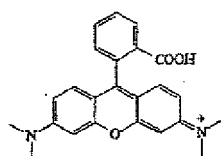
used for activation.

25. The use of a carboxamide dye as claimed in any of claims 1 to 16 for qualitative or/and quantitative determination of an analyte.
26. The use as claimed in claim 25, in which the carboxamide-substituted dye of the formula (I) is coupled to the analyte to be detected or/and to a component of a detection reagent or/and to a support.
27. The use as claimed in claim 25 or 26, in which detection comprises an immunological detection or/and detection by way of nucleic acid hybridization.
28. A conjugate of a carboxamide-substituted dye of the formula (I) as claimed in any of claims 1 to 16 and a binding partner.
29. The conjugate as claimed in claim 28, in which the binding partner is selected from among peptides, polypeptides, nucleic acids, nucleosides, nucleotides, nucleic acid analogs and haptens.
30. The use of a conjugate as claimed in claim 28 or 29 in nucleic acid hybridization processes and immunochemical processes.
31. The use as claimed in claim 26 or 27, in which coupling to the analyte to be detected or/and the component of a detection reagent or/and the support takes place via the substituents  $R_5$  or/and  $R_6$  of the carboxamide-substituted dye of the formula (I), the moieties  $R_5$  and  $R_6$  being as defined in claim 1.
32. The use as claimed in claim 31, in which coupling is carried out via a covalent bond.

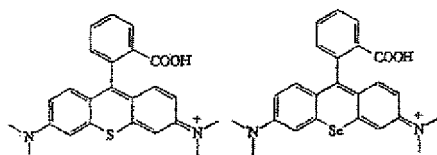
## ABSTRACT

The present invention relates to carboxamide-substituted dyes, the production and use of such dyes as labeling groups in analytics.

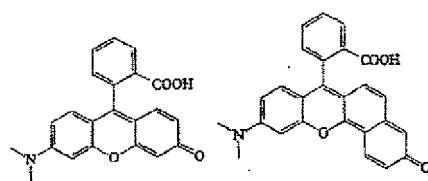
**Figure 1**



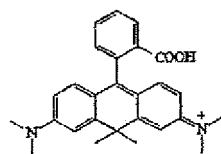
Rhodamines



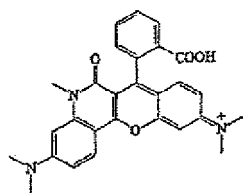
Thiorhodamines,  
selenorhodamines



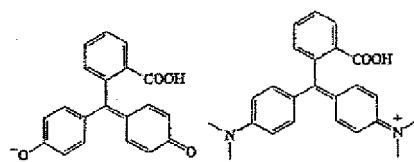
Rhodol dyes



Carbopyronines

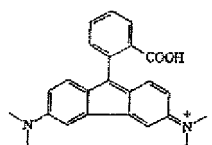


Amidopyrylium dyes

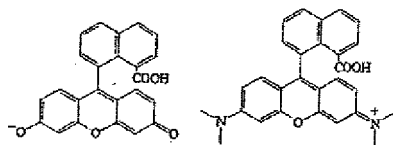


Triphenylmethane dyes

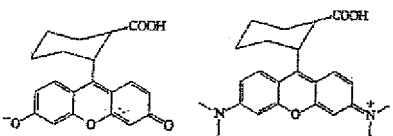
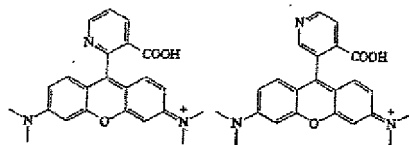
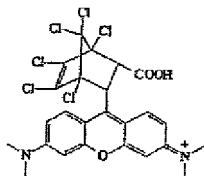
Figure 1 (continued)



Fluorene derivatives



from naphthalic anhydride

from cyclohexanedicarboxylic  
acid anhydridesfrom pyridinedicarboxylic acid  
anhydridesfrom 1,4,5,6,7,7-hexachloro-5-  
norbornene-2,3-dicarboxylic  
acid anhydride

**Figure 2**

Spectral data in ethanol

(s: acidified with 1% trifluoroacetic acid; b: basic with 1% triethylamine)

 $\lambda_a$ : Absorbance maximum $\lambda_f$ : Fluorescence maximum $\eta_f$ : Fluorescence quantum yield

	Structure	$\lambda_a$ / nm	$\lambda_f$ / nm	$\eta_f$ / %
1 NK 50		561	585	48
2 NK 51		536	563	92
3 NK 54		584	606	35
4 NK 56		566, s 622, b	650, b	40
5 NK 57		624	650	88

Figure 2 (continued)

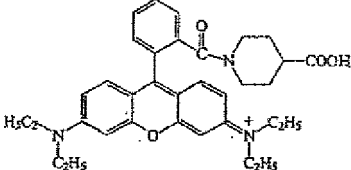
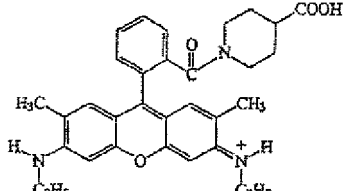
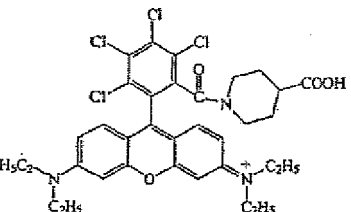
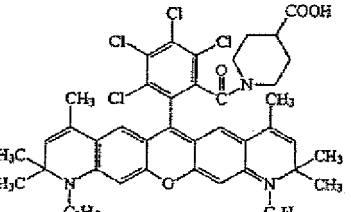
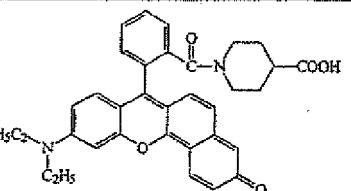
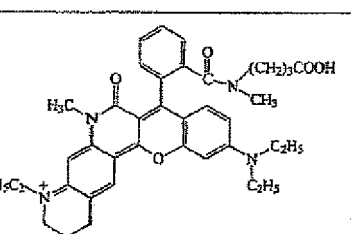
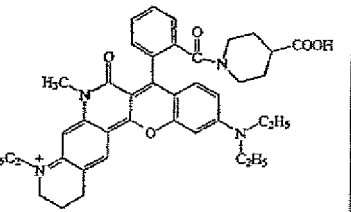
6 NK 58		562	586	46
7 NK 59		535	565	92
8 NK 60		584	605	34
9 NK 61		625	655	89
10 NK 62		565, s 623, b	650, b	40
11 NK 63		615	680	10
12 NK 64		614	677	9

Figure 2 (continued)

13 NK 65		618	650	66
14 NK 66		618	648	65
15 NK 76		563	586	46
16 NK 77		561	585	48
17 NK 78		619	644	69
18 NK 136		512, b	530, b	85
19 NK 106		530	556	20

Figure 2 (continued)

20 NK 80		645, b	700, b	16
21 NK 81		520, b	545, b	80
22 NK 82		624	644	89
23 NK 83		496	519	80
24 NK 107		552	-	0,5
25 NK 84		573	595	92



Figure 2 (continued)

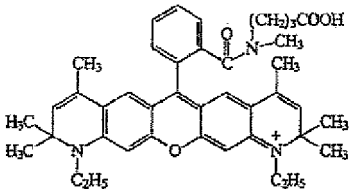
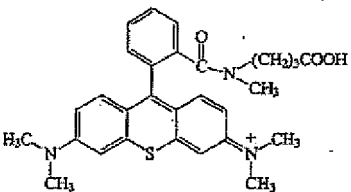
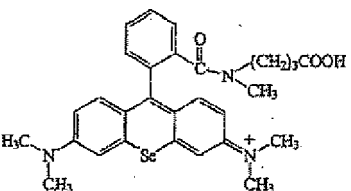
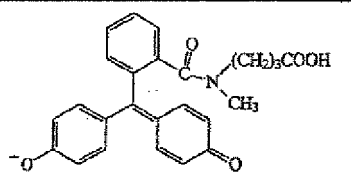
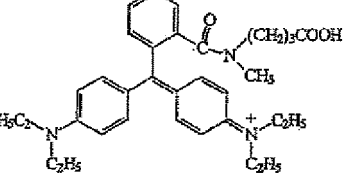
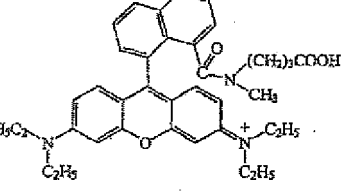
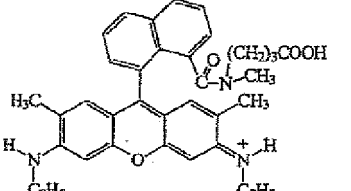
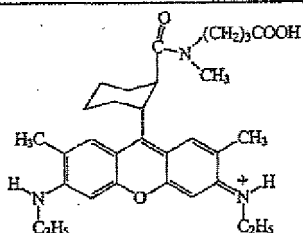
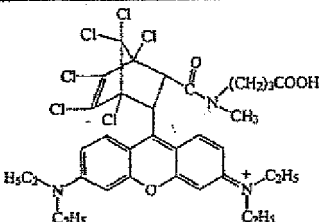
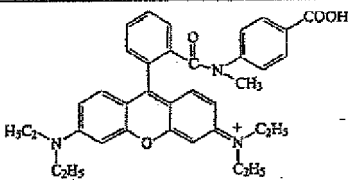
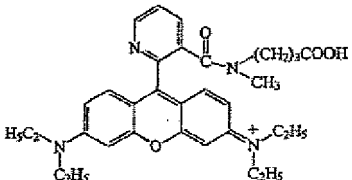
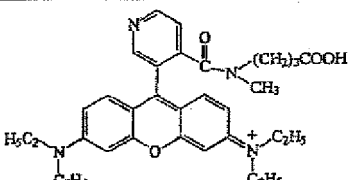
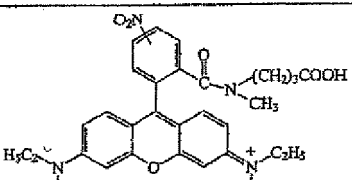
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27 NK 86		575	600	45
28 NK87		580	-	0
29 NK 88		570, b	-	0
30 NK 89		640	-	0
31 NK 90		565	590	55
32 NK 108		538	560	90

Figure 2 (continued)

33 NK 91		575	605	35
34 NK 92		585	615	30
35 NK 109		562	594	80
36 NK 93		581	610	30
37 NK 94		558	580	48
38 NK 95		558	580	48

Figure 2 (continued)

39 NK 110		533	560	87
40 NK 96		570	597	45
41 NK 99		556	578	35
42 NK 102		562	590	45
43 NK 103		562	590	45
44 NK 104		557	575	1



**Figure 3**

Spectral data in ethanol

 $\lambda_a$ : Absorbance maximum $\lambda_f$ : Fluorescence maximum $\eta_f$ : Fluorescence quantum yield

	Structure	$\lambda_a$ / nm	$\lambda_f$ / nm	$\eta_f$ / %
46 NK 47		563	588	47
47 NK 48		536	565	92
48 NK 52		585	607	34

Figure 3 (continued)

49 NK 53		626	648	87
50 NK 55		562, s 623, b	650, b	40
51 NK 67		561	585	46
52 NK 68		563	585	47
53 NK 70		563	584	46
54 NK 71		559	583	45
55 NK 97		562	586	47

Figure 3 (continued)

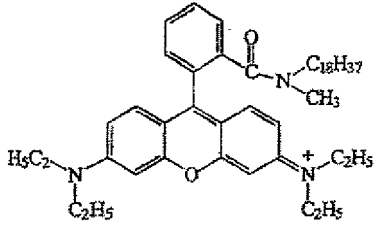
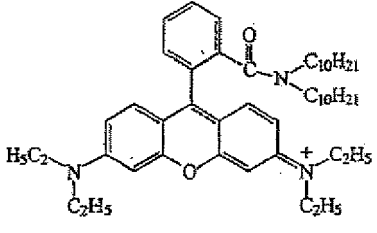
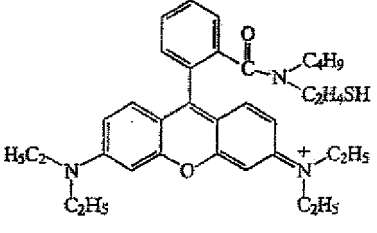
56 NK 98		563	586	48
57 NK 100		562	585	48
58 NK 101		562	585	48

Figure 4

	Structure
59 NK 69	
60 NK 72	



Figure 5

NK 51

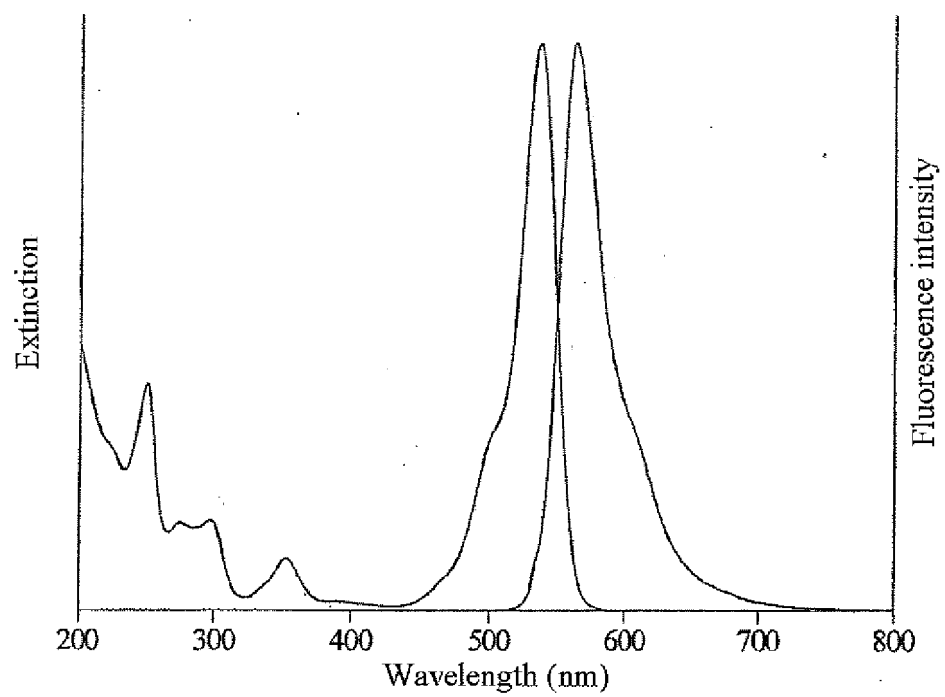
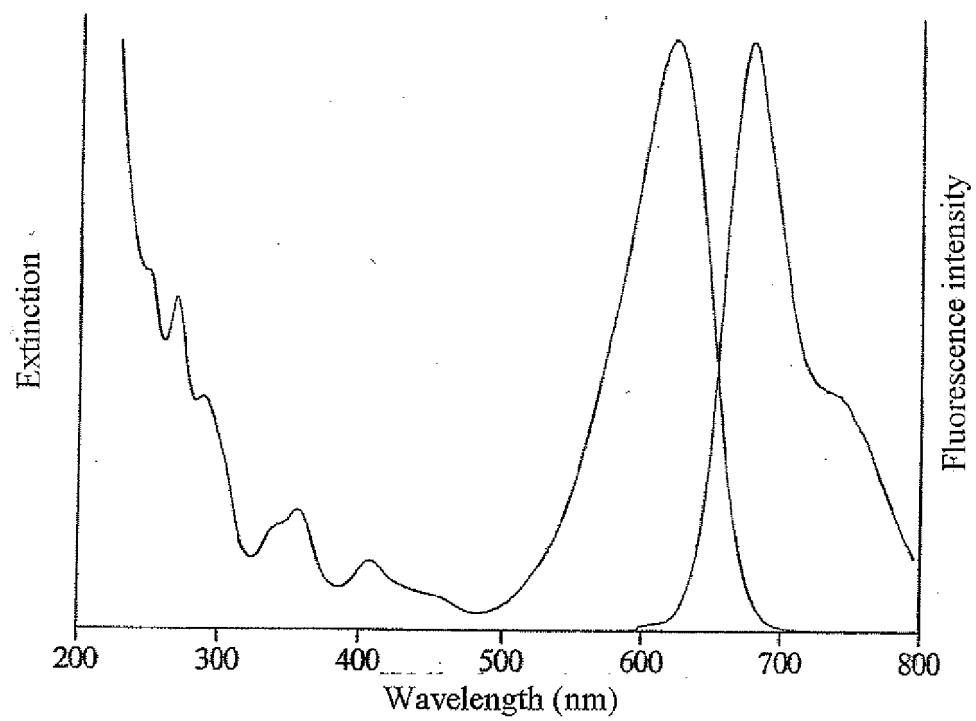


Figure 6

NK 56



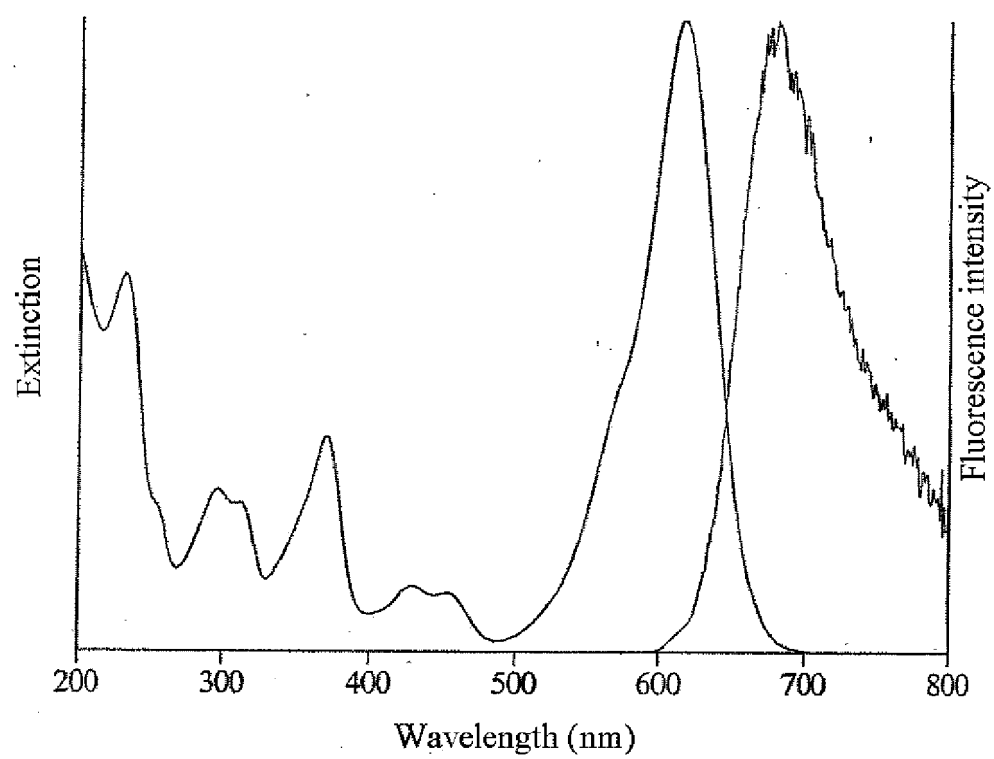
**Figure 7****NK 63**

Figure 8

NK 65

